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Structure and spectral sensitivity of photoreceptors of two anchovy species: *Engraulis japonicus* and *Engraulis encrasicolus*

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ABSTRACT

The morphology, fine structure and spectral sensitivity of retinal photoreceptors of two anchovy species were investigated using light and electron microscopy and microspectrophotometry. Distinct regional specialisation of cones was observed. Long and short (bilobed) cones were observed in the horizontal retinal belt, including the nasal and temporal retinal zones. Only triple cones with two long lateral components, one small central component were observed in the dorsal and ventro-nasal retinal regions. The long cones presented various lamellar organisation patterns: (1) in parallel along the cell axis in the central retina, (2) oriented transversely at the base of the outer segment, and (3) tilted longitudinally while extending to the tip of the cone in the retinal periphery. In the short cones, the lamellae were always oriented along the cell axis, and their planes were perpendicular to the lamellae in the long cones, providing a structural basis for the detection of polarisation of incident light. The lamellae in all the outer segments of the triple cones are arranged perpendicular to the long cell axis. In both species, the long and short cones from the ventro-temporal retina were slender and more densely packed, and the outer segments of the long cones lay far more sclerad compared with the outer segments of the bifid cones. Microspectrophotometry revealed that in both species the lateral components of the triple cones displayed a maximum absorbance wavelength (λ_{max}) of approximately 502 nm, while the short central components were more shortwave sensitive (λ_{max} = 475 nm). The λ_{max} of all long and short cones in the ventro-temporal zone was 492 nm, compared to 502 nm in other retinal regions. Anchovies are unique among vertebrates in that they contain clear structural basis for both colour and polarisation vision in the same retina.

1. Introduction

The structure of the retina is common to all vertebrate species: rods and cones of several types are arranged in a continuous layer with a distinct morphology and complement of visual pigments in the numerous photoreceptive membranes (lamellae) of the outer segments. Changes in illumination result in the excitation of the photoreceptors and secondary neurons that process primary visual information. It has been suggested that changes in retinal arrangement and morphology are closely related to peculiarities of the specific visual world and tasks of most animals (Lythgoe, 1979; Munz & McFarland, 1977).

The morphologies of the retinas of many representatives of the Engraulidae family are significantly different from the morphologies observed in all fish and other vertebrate species. The short and long cones of anchovies are interlocked in specific units known as "polycones" (Fineran & Nicol, 1978), which are arranged in rows that alternate with rows of rods (Heß et al., 2006; Zueva, 1981). The outer segments of the short cones are bilobed (and sometimes termed "bifid"); in contrast with the transverse pattern observed in other vertebrates, the layers of the lamellae of each cone lobe lie along the cell axis. The long cones are also unusual compared to the cones of other fish; their lamellae are also oriented along the cell's long axis in at least part of the outer segment volume. Moreover, the lamellae of the short and long cones are positioned at right angles to each other, thus providing a morphological basis for polarisation vision (Fineran & Nicol, 1978; Novales Flamarique & Hawryshyn, 1998; Novales Flamarique & Hárosi, 2002; Zueva, 1981).

In addition to short and long cones, some anchovy species have specialised triple cones, described for the first time in European anchovy, *Engraulis encrasicolus* (Zueva & Govardovskii, 1991). The triple cones comprise three photoreceptor components that are bundled together in a group, and appear as a succession of rows in limited zones of the dorsal and ventral retina, where they exhibit a clear regional specialisation. The lamellae of the outer

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segments of the triple cones in species of the genus *Engraulis* are oriented "normally", i.e. transversely to the cell's long axis (Heß, 2009; Novales Flamarique, 2011; Zueva & Govardovskii, 1991). However, not all species of anchovy have triple cones. Despite a thorough investigation of the spatial distribution of cones across the entire retina, the presence of triple cones was not detected in the Bay anchovy, *Anchoa mitchilli* (Fineran & Nicol, 1978; Novales Flamarique & Hárosi, 2002). A study of the retina of the Japanese anchovy, *Engraulis japonicus*, also did not show the presence of triple cones (Awaiwanont et al., 2001). However, other closely related species, such as *E. encrasicolus* and *Engraulis mordax*, have triple cones.

Notably, detailed investigation of the morphology and ultrastructure of the anchovy retina (Fineran & Nicol, 1978; Heß, 2009; Heß et al., 2006; Novales Flamarique, 2011; Zueva, 1981; Zueva & Govardovskii, 1991), the spectral sensitivity of anchovy photoreceptors and the properties of anchovy visual pigments have only been studied by microspectrophotometry (MSP) in the European anchovy (Zueva & Govardovskii, 1991) and the Bay anchovy (Novales Flamarique & Hárosi, 2002). MSP has shown that the outer segments of rods, the short and long cones of the European anchovy have the same λ_{max} and their absorbance spectrum matches rhodopsin template. In contrast, members of the triple cones contain different visual pigments (Zueva & Govardovskii, 1991), potentially providing the basis for colour discrimination in the dorsal and ventral retinal areas. However, these conclusions were based upon the estimation of the half-bandwidth of spectral absorbance data, which provides insufficient information about the nature of visual pigments in these species.

A comparative light microscopy study of museum anchovy specimens revealed a high level of diversity within the retinal structure of 11 anchovy species (Heß et al., 2006). Moreover, detailed studies of the retinal ultrastructure in a limited number of species have yielded contradictory data on the orientation of the lamellae in the long cones (Awaiwanont et al., 2001; Fineran & Nicol, 1978; Novales Flamarique & Hárosi, 2002; Zueva, 1981). The species of the family Engraulidae dwell in various ecological niches, such as seas, estuaries and freshwater basins in temperate, subtropical and tropical zones. Thus, considering the unique organisation of the retina in certain species of anchovy and the inconsistencies found in key parameters, more comprehensive research is necessary. The goal of this study was to investigate the morphology, ultrastructure and spectral sensitivity of retinal photoreceptors in two species of the genus *Engraulis*.

2. Materials and methods

2.1. Fish

Adult Japanese anchovies, *E. japonicus*, were obtained in summer months between July 2008 and August 2011 in the Vostok Bay (Sea of Japan) near Marine Biological Station "Vostok" of the A.V. Zhirmunsky Institute of Marine Biology FEB RAS. The fish were caught at night using a fixed net, placed immediately in a closed thermos containing melted seawater ice and delivered to the laboratory within a half an hour for use in morphological and histological analyses and microspectrophotometry. The fish were immobilised in seawater containing a high concentration of the anaesthetic MS222 (Sigma) and subsequently decapitated. The eyes were enucleated and dissected in small Petri dishes placed on ice under a stereomicroscope.

The fish were treated in accordance with the EU Directive of 2010/63/EU, and the Scientific Council of the Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences (IMB FEB RAS) approved the experimental procedures. The details of the retina of the European anchovy (Black Sea subspecies) *E. encrasicolus ponticus* were described using a collection of semi-thin sections and TEM micrographs, which were obtained in earlier studies (Zueva, 1981; Zueva & Govardovskii, 1991).

2.2. Histology

Extracted eye cups were fixed for several days in a 2% paraformaldehyde/2% glutaraldehyde solution at 4 °C, post-fixed in a 1% osmium tetroxide solution and embedded in an Epon–Araldite mixture. Semi-thin radial and tangential sections (1 μm) of pieces of eye cup, which were precisely oriented relative to the embryonic fissure, were obtained using an ultramicrotome (LK 2B), stained with a 1% toluidine blue solution, and examined under a Olympus BH2 light microscope. Ultrathin sections contrasted with lead citrate were examined under JEM 100B (JEOL) and Zeiss Libra 120 transmission electron microscopes.

For microspectrophotometry and *in situ* studies of the photoreceptors using a light microscope, each eye cup was placed onto a Petri dish containing a chilled physiological saline solution (0.9% NaCl solution in 0.06 M phosphate buffer, pH 7.2) for retinal extraction. A small slice of the retina, which was free of pigmented epithelium, was torn up into tiny fragments using sharp needles on the glass slide in a few drops of physiological saline solution. Afterwards, a drop of methylcellulose (m.v. 4000) solution was added to 1–2 drops of the saline solution containing the isolated photoreceptors to increase the viscosity of the medium and prevent spontaneous movement during microscopic observation. The mixed solution containing suspended photoreceptors was placed between two cover glasses, sealed with Vaseline, and examined under a POLYVAR light microscope (Reichert-Jung, Austria) equipped with Normarski optics or used for microspectrophotometry. Digital photos were obtained using a Canon S50 camera with a Leica DC150 camera adapter.

2.3. Microspectrophotometry

Retinal fragments from 16 fish specimens of *E. japonicus* were used. The eyes were dissected on ice in infrared light (4-LED array) under a stereomicroscope equipped with a high-resolution analogue video camera (WATEC Co., Korea). The image from the video camera was controlled using a black-and-white monitor covered with dark red acrylic glass.

The absorption spectra of the outer segments of photoreceptors, which contain the visual pigments, were measured using a microspectrophotometer equipped with a specially designed "jumping" table (Govardovskii & Zueva, 1988). This is single-beam device; the measured object is placed on the table vibrating at small amplitude and 30 Hz frequency. The measuring beam penetrates alternatively the object and near-cell free area, and electronics makes all next processing. The same device was used during MSP studies of fishes of the Lake Baikal (Bowmaker et al., 1994). It includes a MBR light microscope, a MDR grating monochromator (both instruments were obtained from LOMC, St.-Petersburg, Russia) and a registering attachment with a photomultiplier FEU-79 and an amplifier. A dry quartz-mirror condenser UF 40×0.5 and a 40×0.95 dry objective (both from LOMC) were used. The measuring beam dimensions varied from 2×10 μm to 1×2 μm, depending on the size of the outer photoreceptor segment measured. In some experiments the measuring beam was linearly polarised in the plane of the membranes in the discs of the outer segments using a glass polarisation light filter to enhance the recording.

We measured the absorbance spectra of 190 components of the isolated cones (34 short and 80 long components, and 40 lateral and 36 central components of the triple cones), and 12 groups of rods; the small rod diameters prevented us from obtaining

accurate measurements of single cells. The measurements were conducted in the range of 360–750 nm, with a 1-nm interval. To minimise distortion, the absorption spectrum of one cell was registered only once, starting at 750 nm, and the time sweep across the spectrum was 20 s. An enhanced signal from the photoamplifier was transmitted to an AC/DC converter connected to a personal computer and recorded as a text file.

Further processing of the experimental data included summation and averaging of raw recordings from many cells of each type using the software TableCurve2D (SYSTAT Software Inc.) and MSPPROC; general principles of the function have been previously described (Govardovskii et al., 2000; Kondrashev, 2008). Use of the latter software made it possible to adjust zero levels, to smooth the data using several known algorithms and approximate the experimental data using calculated templates for visual pigments based on Vitamin A (rhodopsin, porphyropsin) (Bowmaker, 1995; Govardovskii et al., 2000).

3. Results

3.1. Photoreceptors: morphology and electron microscopy

Zones containing various complements of cones were identified in the retinas of both species. Each zone contains parallel rows of densely packed cones. In the dorsal and ventral regions of the retina, the rows consist exclusively of triple cones, but in the central, nasal and temporal regions of the retina, the rows are composed of alternating long and short cones (Figs. 1A, B and H, 2A). The cones are arranged into continuous rows, and each complement of cones changes at the border between zones. The chain of triple cones terminates simultaneously in all the adjacent rows and transforms into a succession of long and short cones (Fig. 1A). Numerous thin rods occupy the retinal space between the rows of cones. The outer segment is approximately 1 μm thick, and the lamellae are arranged normally in tangential sections, creating a typical undulate pattern (Fig. 2H).

When isolated from the retina, the long and short cones are always concatenated (Fig. 1C–F), often with attached pigment cells inserted between the cone outer segments (Fig. 1E and F). The pigment cells contain melanin and guanine granules and crystals. The walls of the pigment cells border the outer segments and partially contact the ellipsoids of the long cones; the vitreal sharp end of each pigment cell inserts between the lobes of the bifid short cone (Fig. 1E and F, 2A and D). Radial positions of different cone types observed in the TEM sections are characterised by distinct differences in the structure and packing of the mitochondria in the inner segments (Fig. 2F and G).

The mechanical strength of the "polycones" (*sensu* Fineran & Nicol, 1978) is maintained by deep penetration of the outer segments of the short cones into the ellipsoids of adjoining long cones. Bilayered cisternae were observed in the contact zone, under the double-layered membrane of each ellipsoid; they do not underlie the ellipsoid membranes facing the rods (Fig. 2F and G).

Notably, cones from different retinal zones differ in morphology and design. The long and short cones isolated from the ventro-temporal zone (temporal to the embryonic fissure) are much more slender than the cones from the central retinal zone (Fig. 1C and F vs. D). In the central and the nasal retina, the ellipsoids of all the cones are positioned almost at a single level (Fig. 1D); they appear as a single layer in the radial section. In the ventro-temporal zone, in contrast, the short cones are displaced vitreally relative to the long cones. The upper tips of the short cones are positioned at the level of the vitreal margin of the ellipsoids of the long cones (Fig. 1C); thus, the short and long cones appear as a double layer of ellipsoids. The wedge-like processes of pigment cells containing guanine platelets are significantly elongated and fully cover the outer segments of the long cones and their ellipsoids (Fig. 1C and D). Therefore, they reach a bifurcation point in the outer segments of bilobed cones (Fig. 2D), which is a pattern observed in other retinal areas.

The layout of the lamellae is distinguishable even under a light microscope at high magnification (Fig. 1C and D). The lamellar planes of the short and long cones in the isolated polycone were mounted on a glass slide such that the long axes of both cell types were parallel with the plane of the glass (Fig. 1C and F). It was impossible to follow the orientation of the lamellae along the entire length of the outer segment because its apical region usually becomes detached during preparation; thus, the arrangement of the lamellae in the basal and apical regions could differ. In cones obtained from the ventro-temporal zone they were aligned along the long cell axis or across the axis in the other zones.

The triple cones of the Japanese anchovy and other congeneric species (Heß, 2009; Novales Flamarique, 2011; Zueva & Govardovskii, 1991) are composed of two large lateral components, with one much shorter and thinner central component between them (Figs. 1G–I and 2J). During retinal preparation, these components were separated as a single triple unit, often bearing long myoids (Fig. 1G), but they never joined each other in a chain, as observed for long and bilobed cones. The outer segment of the central component is significantly shorter than the outer segments of the lateral components and is situated at the level of their bases (Figs. 1H, I and 2I). The lamellae in all the outer segments of the triple cones are arranged perpendicular to the long cell axis (Fig. 2I and K).

Despite the morphological variations observed in different retinal zones, the cones we studied have one common feature, i.e., the existence of accessory outer segments, which was first described in the mid-1950s (Engstrom, 1960). The additional outer segments appeared as long cytoplasmic protrusions that run almost up to the scleral tips of cones of all types and are connected to the "main" outer segment through cytoplasmic bridges (Figs. 1I and 2C and I).

3.2. Visual pigments

3.2.1. Japanese anchovy (*E. japonicus*)

The spectral absorbance of the long and short cones isolated from retina fragments outside the ventro-temporal zone has λ_{max} at approximately 502 nm. Notably, experimental data could not be fitted by the template for a single pigment. The same is true for lateral members of triple cones (Fig. 3A). According to Toyama et al. (2008) the Japanese anchovy has only Vitamin A_1-based pigments, but the experimental points of the longwave slope lie far above the rhodopsin template. We did not attempt to obtain approximation of the data to any combination of the templates because there are no reliable data in the literature concerning the chromophores or opsins of visual pigments in anchovies.

The spectral sensitivity was the same for the long and the short cones obtained from the ventro-temporal retinal zone but differed from that of both cone types from other retinal zones. The absorbance spectrum best fit the template for the single rhodopsin pigment at λ_{max} = 492 nm (Fig. 3C and D).

We performed a number of experiments using differential bleaching to determine if a pigment mixture was present in the short cones. After recording the initial ("native") curve, the outer segment of the cone was bleached for 40–60 s using a monochromator beam at $\lambda \approx 610$ nm, which is in the range of absorbance of a presumptive long wave-sensitive component of the pigment mixture but out of the range of the spectral absorbance of the presumptive short wave-sensitive component. After bleaching, an additional recording from the same outer segment was performed, and the difference between the two data sets gave the absorbance

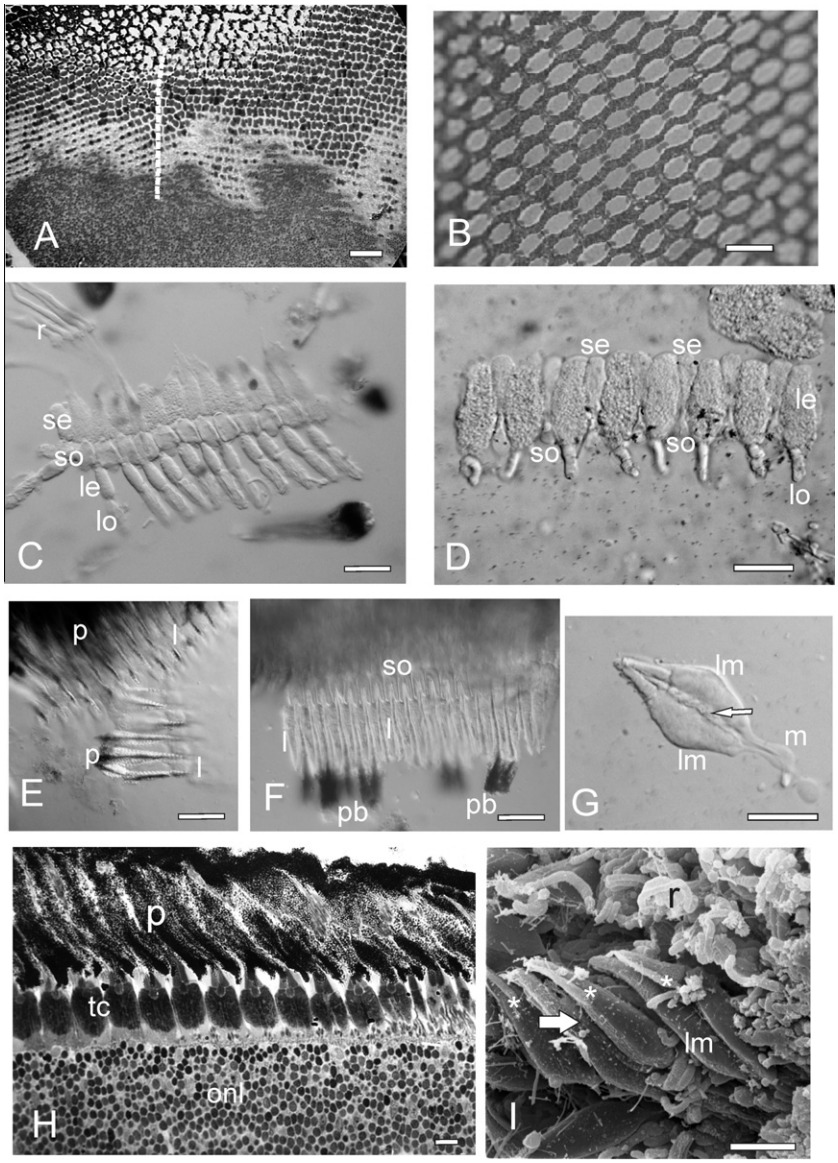

Fig. 1. Photomicrographs of cones of *E. encrasicolus* (A, H, and I) and *E. japonicus* (B–G). A, B, and H – semithin sections. (A) Tangential section of cone rows at the level of ellipsoids and outer segments in the transition zone (dash line) between polycones (left) and triple cones (right). (B) Tangential section of rows of polycones at the level of ellipsoids of long cones (light ellipses) and outer segments of short (bilobed) cones. The rows of polycones alternate with the dark rows of rods. (C) Lateral view of part of a polycone from the ventrotemporal retina. Note the distance between the outer segments of long and short cones relatively to the same structures in the polycone unit from the central retina (D), se: short cone ellipsoids, so: short cone outer segments, le: long cone ellipsoids, lo: long cone outer segments, r: rods. (D) Lateral view of part of a polycone from the central retina, designations see (C). (E) Lateral view of two parts of a polycone row from the central retina. Note long processes of pigment cells with guanine platelets separating adjoining long cones, p: pigment cells, l: long cones. (F) Lateral view of a polycone row from the central retina. Note the tips of processes of the pigment cells penetrating points of bifurcation of the lobes of short cones, pb: pigment cell body, so: short cone outer segments; l: long cones. (G) Isolated triple cone. lc: lateral component, arrow: central component, m: cone myoids. (H) Radial section of triple cone row in the dorsal retina, p: pigment epithelium, tc: triple cones, onl: outer nuclear layer. (I) Scanning electron microscopy, a group of triple cones. lc: lateral component, arrow: central component, asterisk: accessory outer segment, r: rods. Scale bars: A = 40 μm; $B - I$ = 10 μm.

of the long-wave pigment. Both components presented spectral properties indicative of the presence of two pigments with putative maxima at 490 and 510 nm (Fig. 3E), but we cannot definitively confirm the identify of these pigments until their biochemical natures have been sufficiently well defined.

The spectral absorbance of the central member of the triple cones obtained had λ_{max} of 475–480 nm and did not fit a single pigment template (Fig. 3B). Attempts to obtain the best result were unsuccessful: the experimental data did not match any appropriate solution for the long-wave slope of the template.

The rods of the Japanese anchovy had a λ_{max} of 502 nm. The value was obtained from groups of several overlapping rods; a single rod is thin (often less than 1 μm in diameter), which makes correct measurement of the properties of one rod difficult. The data show a good fit for a rhodopsin template and do not indicate admixture with any other pigments (Fig. 3F).

3.2.2. European anchovy (*Engraulis encrasicolus*)

Zueva and Govardovskii (1991) were the first to publish data concerning the spectral sensitivity of retinal photoreceptors in the European anchovy. The conclusions of the authors concerning the nature of visual pigments are primarily based on the width of the spectral curves. We recalculated their results using a better template approximation method that was generated using the software applied in the present work (see Section 2).

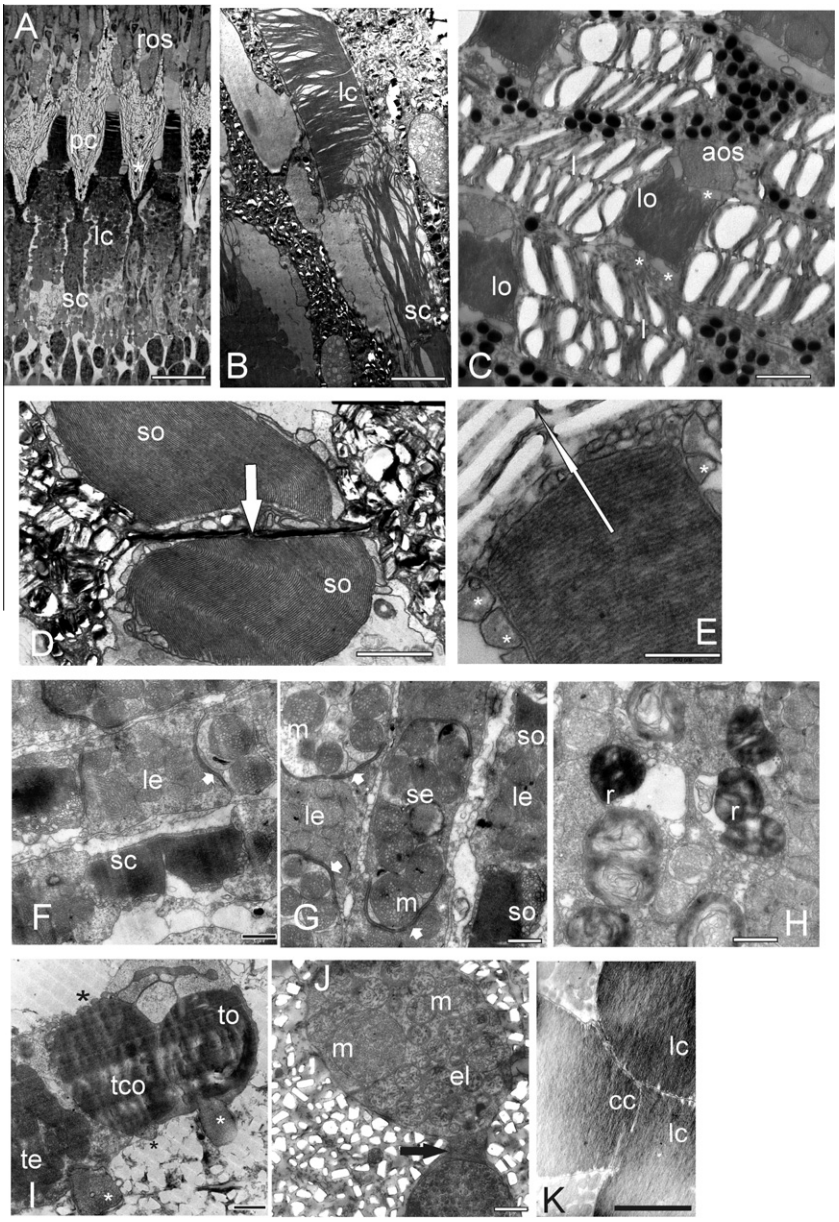

Fig. 2. TEM-micrographs of radial (A, B, and K) and tangential (C–J) sections from the retina of *E. encrasicolus* (A, B, D, and K) and *E. japonicus* (C, E–J). (A) Radial section of the cone row showing alternating arrangement of long (lc) and short (sc) cones. Note the insertion of processes of the pigment epithelium cells (pc) into bilobed outer segments of the short cones. asterisk: guanine platelets, ros: rod outer segments. Bar: 5 μm. (B) Radial section showing orthogonal layout of the lamellae in the outer segments of neighbouring long (lc) and short (sc) cones. Bar: 2 μm. (C) Section of the long cone outer segments between the pigment cell processes containing loose lamellae (l) and guanine platelets. asterisks: calycal processes, aos: accessory outer segment, lo: long cone outer segment. Bar: 1 μm. (D) Section of the short cone at the level just after outer segment is splitted. arrow: a tip of the pigment cell process, so: outer segment. Bar: 1 μm. (E) Same as C at greater magnification showing lamellar arrangement transversely to the direction of the cone row (arrow), asterisk: calycal processes. Bar: 0.5 μm. (F) Section of the short cone (sc) at the level of the outer segment base where two lobes begin to split. Long side of the short cone is facing to the ellipsoid of the long cone (le). Arrows – see next legend to G. Bar: 1 μm. (G) Slightly oblique section showing cone rows at the level of alternating ellipsoids of long and short cones in the ventrotemporal retina. Note a specific mitochondria and membrane structures double layer cysternae (arrows) in a contact zone between different type of cones in a row. le: long cone ellipsoid, se: short cone ellipsoid, m: mitochondria, so – short cone outer segment. Bar: 1 μm. (H) The rows of rod outer segments (r), more scleral than in G. Bar: 1 μm. (I) Triple cone, oblique section at the level of the ellipsoid of one lateral component (te) and outer segment of the second lateral component (to) and outer segment of the central component (tco). Black asterisks: calycal processes, white asterisks: accessory outer segments. Bar: 1 μm. (J) Triple cone, section at the level of ellipsoids. el: ellipsoid of the lateral component, arrow: ellipsoid of the central component, m: mitochondria. Bar: 1 μm. (K) Triple cone, section showing transversal position of the lamellae in all outer segments. lc: lateral component, ccm: central component. Bar: 1 μm.

The values calculated for the maximal absorption in the rods, long cones and large lateral components of the triple cones were almost the same as the value previously obtained in the Japanese anchovy (500 nm); therefore, approximation of the data using a single rhodopsin template was also impossible (Fig. 4A). The λ_{max} of the short cones (Fig. 5a in Zueva & Govardovskii, 1991) was approximately 492 nm, which was similar to the value observed for short cones from the ventro-temporal zone of the retina in the Japanese anchovy.

The central component of the triple cones displayed a λ_{max} of 474 nm, which was slightly lower than the value obtained for analogous elements in the Japanese anchovy (Fig. 4A). In this case, the spectral data were better fit to the template of a single rhodopsin pigment, either because the measurements (positioning of the measuring beam) were more accurate or because these cones contain a different complement of visual pigments.

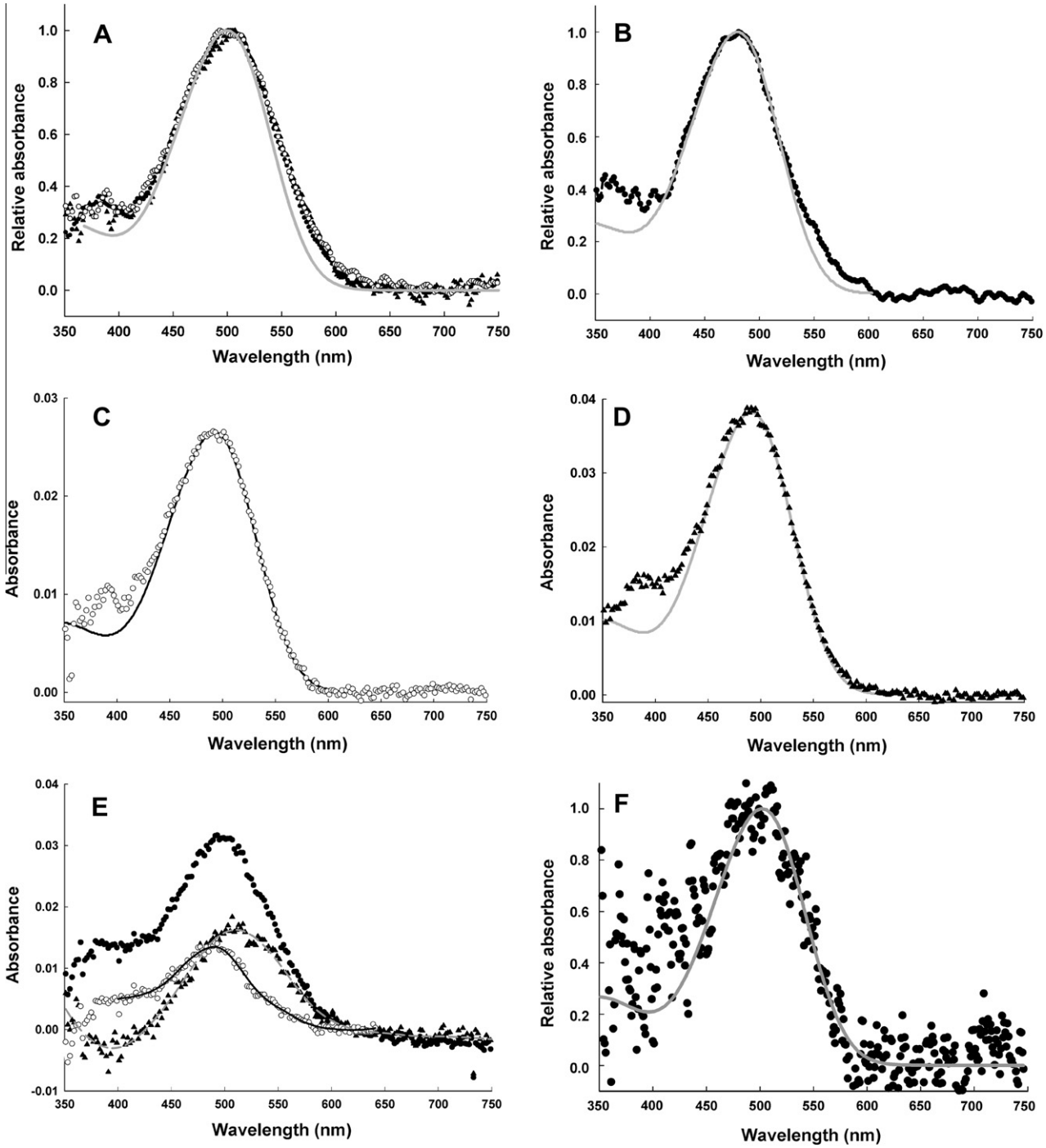

Fig. 3. The absorbance spectra of the photoreceptors of the Japanese anchovy *Engraulis japonicus*. The spectra are the means of records obtained from single cone outer segments (A–E) or from the group of the rod outer segments (F). Symbols are the recorded data, solid lines are the best fit of the rhodopsin pigments template curves (C, D and F), templates of the rhodopsin (λ_{max} = 502 nm) (A), rhodopsin (λ_{max} = 475 nm) (B), and smoothed data (E). The number of records (n) is shown in brackets. (A) Triangles – long cones from central and nasal retina (n = 18), open circles – short cones from central and nasal retina (n = 16), filled circles – lateral components of the triple cones (n = 20). (B) Central component of the triple cones (n = 17). (C) Long cones from the ventro-temporal retina (n = 19). (D) Short cones from the ventro-temporal retina (n = 18). (E) Differential bleaching, short cones from the nasal retina, see Section 3.2.1 for details. Filled circles – first recording (n = 12). Open circles – recording after bleaching using 610 nm light source, triangles – difference between the first and second spectral data. Solid smoothed curves are indicative of the presence of two visual pigments with putative λ_{max} at 510 and 490 nm. (F) Rods (n = 12).

4. Discussion

4.1. Regional specialisation of the retina

Some data concerning the morphology and ultrastructure of the retinal photoreceptors of the Japanese anchovy, except for the description of the triple cones, have been previously published (Awaiwanont et al., 2001). A comparison of the published data (Heß, 2009; Novales Flamarique, 2011) and the results obtained in this study showed that the general and detailed structure of the retina, the spatial distribution of different types of cones and the association with the surrounding pigment cells are similar in the three anchovy species of the genus *Engraulis*.

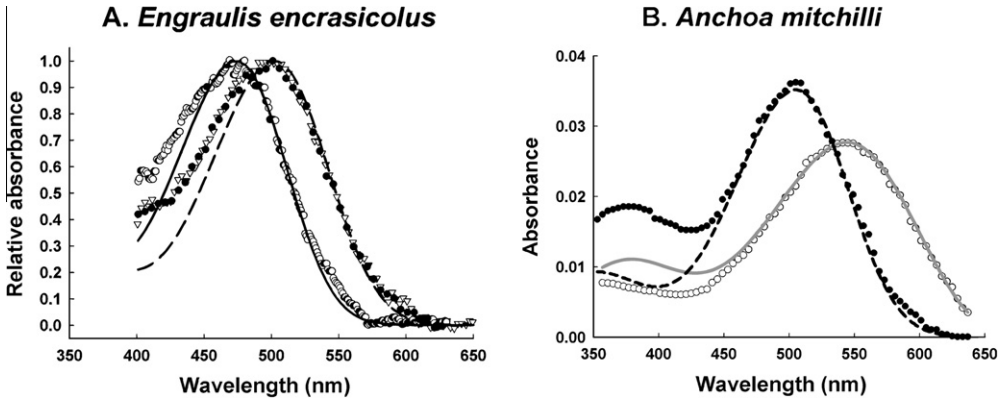

Fig. 4. The absorbance spectra of the photoreceptors of the anchovies *Engraulis encrasicolus* and *Anchoa mitchilli*. The data (symbols) were plotted from published figures using X–Y digitiser (A – Fig. 5 from Zueva & Govardovskii, 1991; B – Figs. 2 and 3 from Novales Flamarique & Hárosi, 2002). (A) Long cones (triangles), lateral components of the triple cones (filled circles) and central component of the triple cones (open circles). (B) Open symbols – long and short cones, filled symbols – rods. Solid lines are the best fitting of the visual pigments templates (Govardovskii et al., 2000): (A) rhodopsin (λ_{max} = 502 nm and 474 nm); (B) rhodopsin (λ_{max} = 502 nm) – dashed line; porphyropsin ((λ_{max} = 543 nm) – grey line.

In the nasal, central and temporal retina, we observed long and short cones, whereas triple cones dominated the ventro-nasal and dorsal retina. In previous studies of the retina in *E. mordax* and *E. japonicus* (Awaiwanont et al., 2001; Fineran & Nicol, 1978; Heß et al., 2006), triple cones were not found, potentially because the retinal regions containing cones of these types are rather small and hard to identify (Heß, 2009; Zueva & Govardovskii, 1991).

The existing discrepancies in the description of the morphology and ultrastructure of long and short cones could result from differences in the retinal regions sampled. It is well known that fish develop distinct regional retinal specialisations with regard to photoreceptor type, morphology, ultrastructure, distribution and spectral sensitivity (e.g. Collin, 1997; Temple, 2011). Baburina (1953) presented the first description of differences in the retinal structures of *E. encrasicolus*. This author focused on the differences between retinal structures in the *area temporalis* and dorso-nasal regions of the retina. Differences between these regions were only observed in terms of the packing density of the cone mosaic, which is much higher in the *area* temporalis (see also O'Connell, 1963 for *E. mordax*). A comparative description of the relative position of the ellipsoids of the long and short cones in both regions is consistent with recent data (see Fig. 1A in Heß et al., 2006; Heß, 2009) and corresponds to our data for the Japanese anchovy (Fig. 1C and D).

The ultrastructure of cones in the anchovies also demonstrates regional features. It was shown in *A. mitchilli* (Novales Flamarique & Hárosi, 2002), *E. encrasicolus* and *E. japonicus* that the lamellae in the long cones are oriented longitudinally throughout the outer segment (Awaiwanont et al., 2001; Heß et al., 2006; this paper). In addition, we have shown that in the Japanese anchovy, this structure is typical only in the outer segments of cones from the ventro-temporal retina including the *area temporalis*. However, there are indications that this lamellar orientation exists only in the apical parts of the outer segments in the retina of the European anchovy (Zueva, 1981).

Taken together, these data suggest that in the long cones from the periphery of the retina, the lamellae in the vitreal part of the outer segment (at a considerable distance from the base) are oriented transversely with respective to the long cellular axis. Closer to the centre of the retina, the lamellae gradually develop a longitudinal orientation. As the anchovy retina grows from the outer margin over the life of the fish (as in other fish species), the lamellae of the recently differentiated cones are oriented transversely at the periphery. A model of this process was recently presented (Novales Flamarique, 2011), but further developmental studies on early embryos and larvae could clarify the details.

4.2. Spectral sensitivity and visual pigments

The spectral sensitivity of the photoreceptors of most anchovy species remains unknown. Before this study, this information was available for only two species (Novales Flamarique & Hárosi, 2002; Zueva & Govardovskii, 1991), and this paper provides additional data for the Japanese anchovy. The rods, long and short cones, and lateral components of the triple cones (except from the ventro-temporal zone) in in both species studied in the present work have the same spectral absorbance with a λ_{max} near 500 nm and experimental data (except for the rods) could not be fitted by the template for a single pigment. In addition, the results of differential bleaching of the short cones in *E. japonicus* give evidence in favour of a pigment mixture. Considering that two studied species are closely related, we find it reasonable to conclude that both types of the European anchovy cones (excluding central components of the triple cones) contain an as yet unidentified mixture of the same pigments as in the Japanese anchovy (Fig. 4A).

The spectral absorbance of the central member of the triple cones also did not fit a single pigment template, especially for the long-wave slope of the curve. We propose two explanations for this result. First, the central components of the triple cones could contain only one visual pigment with a λ_{max} of 475 nm but be slightly contaminated with a small amount of absorbance from the pigments of adjacent lateral components, which have a λ_{max} of 500–502 nm, as a consequence of the overlap between the components. In our microspectrophotometric studies, we used a "jumping" stage, in which the position of the measuring beam changes at a certain frequency; therefore, it is reasonable to suppose that the light beam could come in contact with different closely packed outer segments. Second, the central components could contain a mixture of pigments of unknown spectral properties.

The third (central) component of the triple cones is maximally sensitive to the shorter wavelengths (474–480 nm) in comparison with lateral components (502 nm). Thus, the triple cones could provide colour discrimination in the dorsal and ventral visual fields, consistent with the ability of the *E. mordax* anchovy to discriminate colour stimuli in behavioural (phototaxis) experiments (Loukashkin & Grant, 1965); however, the interpretation of the data is somewhat obscure, suggesting problems with the control of light intensity in natural experiments conducted in voluminous water tanks.

The identification of the spectral curves for the cones in anchovies is difficult because reliable data concerning the biochemical composition of visual pigments are lacking. Information concerning

the nature of the chromophore (retinal$_1$ or Vitamin A$_1$, and retinal$_2$ or Vitamin A$_2$) and the type of opsin is required to characterise the essential mechanism for the determination of the specific spectral properties of visual pigments, as one and the same opsin linked with Vitamin A$_1$ or with Vitamin A$_2$ can produce rhodopsin or porphyropsin, respectively (Bowmaker, 1995). Among the studies concerning the nature of the chromophores of 164 fish species, it was reported that the Japanese anchovy contains pure Vitamin A$_1$ – based rhodopsin (Toyama et al., 2008); however, neither the details of the experiments nor the chromatograms are presented. Therefore, this question needs to be carefully re-examined, as the possibility that another type of visual pigment, porphyropsin, is present cannot be excluded. Our estimation of the published results concerning the visual pigments of *A. mitchilli* (Novales Flamarique & Hárosi, 2002) suggests the presence of an additional pigment.

The spectral absorbance of the rods in *A. mitchilli* is equal to that of the European anchovy and Japanese anchovy, but the long and short cones in the polycones are more sensitive to long wave light, displaying a λ_{max} of approximately 540 nm. After estimating the width of the spectral curve, the authors have concluded that this sensitivity is determined by the presence of rhodopsin; however, our recalculation and approximation of the same experimental data using the known templates (Govardovskii et al., 2000) indicates that curves for the long and short cones were well fitted by the template of pure porphyropsin, with a λ_{max} of 543 nm, at least for a long-wave slope, which is critical in these estimates (Fig. 4B).

Much evidence supports the idea that the majority of marine fish exclusively possess rhodopsin in the retina (Bowmaker, 1995); however, there are more than a few exceptions to this trend, including shallow water species and species dwelling in estuaries and freshened marine waters that possess only porphyropsin or porphyropsin mixed with rhodopsin (Bowmaker, Dartnall, & Herring, 1988; Cummings & Partridge, 2001; Kondrashev, 2008; Kondrashev, 2010; Toyama et al., 2008; White et al., 2004). It is quite possible that *A. mitchilli* and some other species of anchovies also fail to abide by this trend. For instance, among the clupeid fishes, of which anchovies are members, porphyropsin was found to be present in the retinas of *Alosa pseudoharengus* (Schwanzara, 1967), *Dorosoma cepedianum* (Bridges, 1964), estuarine and freshwater anchovy *Coilia nasus* (Toyama et al., 2008) and *Konosirus punctatus* (Toyama et al., 2008; our unpublished data). A$_2$-based visual pigments are more sensitive to long-wave light, which is more abundant in shallow and estuarine waters that contain high concentrations of yellow river outflow (Jerlov, 1976; Lythgoe, 1979). Ecological information shows that the Bay anchovy, in comparison with the European and Japanese anchovies, prefers shallower water, lives much longer in estuaries and downstream reaches, and does not avoid the bottom water layers, judging from the presence of benthic isopods and molluscs in the diet (Allen, Johnson, & Ogburn-Matthews, 1995; Morton, 1989). Thus, the presence of more long-wave-sensitive cones in this species is ecologically advantageous.

In a recent paper, Novales Flamarique (2011) attempted to determine the type of opsins present in the photoreceptors of the Northern anchovy *E. mordax* using immunochemical labelling by mammalian antibodies. This author observed that the lateral components of the triple and long cones were labelled with the same M/LWS opsin antibodies; notably, the short cones were not sensitive to them. This result partially confirms our conclusion that the long cones and large lateral components of the triple cones contain the same complement of pigments; however, the suggestion that the short (polycone) cones, according to our data, contain the same pigments as the long cones remains unconfirmed. Moreover, immunolabelling shows that the short cones, central components of the triple cones and rods were equally labelled by rod opsin (RH1) antibodies, This finding shows at least that the central component of triple cones contains opsin different from that in long and lateral cones, but the labelling of two types of cones by the RH1-specific antibody suggests non-specific binding when mammalian antibodies are applied to the fish. It is possible but very unlikely that there is a significant difference between *E. mordax* and the two congeneric species examined in our study. The proposed presence of pigment mixtures in all cone types creates potential confusion, and it is becoming clear that further progress in evaluation of the visual pigments in anchovies is impossible without molecular genetic studies concerning the existing opsins.

4.3. Mechanisms of polarisation and colour vision in a single retina

Dichromatic system of the triple cones provides photopic colour vision in the dorsal and ventral zones of the anchovy retina. The existence of the retinal regions specialised for colour vision is common in vertebrates (Temple, 2011), including fish (Reckel et al., 2002), amphibians (Firsov, Govardovskii, & Donner, 1994), birds (Maldonado, Maturana, & Varela, 1988) and mammals (Lukáts et al., 2005; Orlov & Podgorny, 2009). However, in anchovies, this specialisation is combined with one additional feature, i.e., the potential use of specific retinal zones in a polarisation analysis as a result of the unusual arrangement of the photoreceptive lamellae in the outer segments of the short and long cones.

Electrophysiological experiments showed that the retina of *E. mordax* (Novales Flamarique & Hawryshyn, 1998) is sensitive to polarised light because of the presence of cones with lamellae positioned along the cell axis (direction of the incoming light) and a multilayered structure of reflective guanine platelets in adjoining V-shaped pigment cells. According to the proposed model (Novales Flamarique & Hawryshyn, 1998), linearly polarised light that penetrates the retina is first absorbed by the long cone outer segment lamellae and then again after reflection from the interference mirrors of the guanine platelets. This scheme focuses on the entire volume of the retina containing polycones. As we have shown, the outer cone segments in the *area temporalis* are displaced at a considerable distance from one another and the reflecting guanine layer covers not only the outer segments of the long cones but also its ellipsoids (Fig. 1E and F). This arrangement could potentially diminish light scattering and losses along the pathway between the outer segments of the long and short cones, thus enhancing the detection of polarisation contrast. More dense packing (about two times) of the rows of cones in the lower part of the ventrotemporal retinal quadrant behind the embryonic fissure (Koch, Seebacher, & Heß, 2010) enhances the acuity of polarisation vision in the part of the retina that faces forward and towards the water surface; thus, this feature could be important for orientation.

It was predicted and observed in insects that a mixing of the signals from the photoreceptors possessing colour and polarisation sensitivity in a single cell (ommatidium) would produce false colour phenomenon (Bernard & Wehner, 1977; Kelber, Thunell, & Arikawa, 2001). "Colour-blind" polycone regions and triple-cone "colour-sensitive" regions are separated in the same anchovy retina what is important not to confuse polarisation and colour vision. It is well known that insects use polarisation for orientation by the polarised sky map (Labhart & Meyer, 1999). Polarisation "detectors" in anchovies and insects display striking similarities with respect to the presence of specialised zones (they face the dorsal visual field) on the retina and in a complex eye and the monochromacy of the polycones and ommatidia, which comprise two receptors with orthogonally oriented light-sensitive lamellae (the planes of which match the direction of physiological illumination). Thus, the part of the anchovy retina that is filled with rows of long and short cones could be responsible for orientation to sunlight, which produces polarisation patterns on the surface and in the water body. It is also possible that anchovies use polarisation sensitivity

for orientation toward potential prey. For example, the Japanese anchovy forages mainly on zooplankton (Tanaka et al., 2008); the chitinous exosceleton of planktonic organisms selectively absorb and reflect polarised light (Novales Flamarique & Browman, 2001), which could in this case, create additional contrast and thus increase the probability of detection of this prey. Also a negative polarisation contrast may arise in the sea due to unpolarized downwelling light that has been scattered from the potential prey viewed against the darker and polarised horizontal background light (Johnsen, Marshall, & Widder, 2011). Thus, the structure and spectral sensitivity of photoreceptors could be essential for the life and behaviour of the common commercial fish species the Japanese anchovy.

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